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EFFICIENT PRODUCTION OF MUTANT PROTEASES

INTRODUCTION

5

Technical Field

The present invention relates to a method for the production of mutant proteins using recombinant DNA techniques. More specifically, the invention relates to the 10 efficient production of protein engineered Bacillus proteases using a homologous Bacillus host strain, from which the capacity to produce the corresponding unmodified protease has been eliminated.

15

Background of the Invention

Bacilli are widely used for the production of industrially important enzymes such as α -amylases, neutral proteases and alkaline (or serine) proteases, see, for 20 example, Debabov: "The industrial use of Bacilli" in: The Molecular Biology of Bacilli, Acad. Press, New York, 1982.

The main advantages of using Bacilli are the large quantities of protein secreted, their lack of production of hazardous substances and their long history of safe 25 industrial use. Several species of Bacilli have therefor been approved for the production of proteins destined for food applications.

The use of Bacillus subtilis as an expression host was demonstrated by Palva et al., Gene 19 (1982) 81-87, 30 describing the expression of the α -amylase gene from B. amyloliquefaciens in B. subtilis. Other references describing heterologous gene expression in B. subtilis are, e.g., Fahnstock and Fisher, J. Bacteriol. 165 (1986) 796-804, who showed the expression of the Staphylococcus aureus 35 Protein A gene; Schein et al., Biotechnology 4 (1986) 719-725, disclosing the expression of human interferon- α 2; and Wang et al., Gene 69 (1988) 39-47, showing the expression

and secretion of human atrial natriuretic α -factor. To ensure the proper secretion of these heterologous proteins the former two references used the α -amylase signal sequence and the latter reference the signal sequence of the B. 5 subtilis subtilisin gene (aprE) fused to the gene to be expressed.

A major problem of Bacillus strains as production hosts for recombinant proteins is that they produce and 10 secrete a variety of proteases which tend to degrade the heterologous proteins produced. See, e.g., Old and Primrose, "Principles of Gene Manipulation", 3rd ed. (1985) 289, Blackwell, Oxford.

In International Patent Application (PCT) WO 89/04866 15 the importance of extracellular proteases is pointed out; about 90% of the proteolytic activity is attributed to the neutral metalloprotease (npr) and the serine protease (apr).

Various solutions have been proposed to overcome this degradation problem.

20 Kawamura and Doi, J. Bacteriol. 160 (1984) 442-444, disclose a B. subtilis double mutant deficient in both extracellular neutral and alkaline protease. First B. subtilis DB100 was made neutral protease negative by transferring the nprR2 and the nprE18 mutations from B. 25 subtilis NT18. A 178 bp fragment containing a part of the apr gene was then deleted by gene conversion.

Fahnestock and Fisher, Appl. Environ. Microbiol. 53 (1987) 379-384, describe the construction of an apr B. subtilis strain starting from a npr strain. They used a 30 plasmid vector containing the apr gene and introduced the cat gene both as an inactivator and a selectable marker.

Sloma et al., J. Bacteriol. 170 (1988) 5557-5563, detected and sequenced a third extracellular protease gene (epr) from B. subtilis. Partial deletion of this gene showed 35 that it contributed only marginally to the extracellular protease activity.

However, none of the referenced B. subtilis strains was completely devoid of extracellular proteolytic activity.

In several publications the production of mutant 5 proteases is described by Bacillus subtilis host strains which are incapable of expressing the wild-type protease gene, thus avoiding mixtures of mutant and wild-type proteases.

In EP-A-0246678 a B. subtilis strain is disclosed 10 which is incapable of secreting enzymatically active subtilisin or neutral protease, but which expresses heterologous protease genes isolated or derived from B. amyloliquefaciens. Site-specific saturation mutagenesis was performed on the B. amyloliquefaciens gene and the mutants 15 were expressed. The negative background of the B. subtilis host strains was obtained by partial deletion of either or both of the original protease genes, or by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis. It was further stressed that the B. subtilis host described is normally 20 sporulating and that asporogenous mutants are unsatisfactory for the production of (recombinant) proteins.

In WO 86/01825 Bacillus strains are described, in particular B. subtilis, with reduced extracellular protease levels. The alkaline protease gene is inactivated by in 25 vitro insertion of a gene coding for a protein which confers a phenotypic trait on the microorganism. The plasmid vector containing the inactivated protease gene is transformed to B. subtilis and the inactivated gene replaces the native chromosomal gene by homologous recombination. No expression 30 and secretion of heterologous proteins is suggested.

WO 88/8033 discloses subtilisin analogs from subtilisin Carlsberg, subtilisin DY, subtilisin BPN', an apr subtilisin from B. subtilis and a subtilisin from B. mesentericus. These mutants are obtained by replacing one or 35 more amino acids in or near the calcium binding site with negatively charged amino acids (e.g. Asp or Glu). The mutants are expressed in B. subtilis.

WO 89/04866 discloses a B. subtilis strain with low secreted protease activity, which was made by a spoOH mutation in an apr⁻npr⁻ double mutant. The obtained triple mutant apr⁻npr⁻spoOH⁻ showed very low secreted protease 5 activity.

The references discussed above describe the expression of homologous and heterologous genes in B. subtilis and the construction of protease-negative B. subtilis strains. It has appeared that protease-negative strains 10 made by partial deletion of the protease gene or by insertion of another gene in the protease gene show several drawbacks, e.g.:

- the original gene may be reactivated. If a gene which is homologous to the inactivated chromosomal gene is 15 introduced into the cell (on a plasmid), homologous recombination can lead to reactivation of the gene.

- the original gene, although inactivated, may lead to the production of partial expression products, when the promoter region is still active. This is disadvantageous in 20 terms of metabolic efficiency.

- the introduction of unwanted phenotypical traits, e.g. antibiotic resistance, depending on the inserted gene.

- plasmid instability, due to homology between a genomic sequence and a part of the inserted plasmid.

25 It is therefore desirable to delete all sequences with possible homology between the chromosome and the plasmid.

There are also other drawbacks for the production of alkaline proteases or other enzymes in B. subtilis. Although 30 Wells et al., Nucleic Acids Res. 11, (1983) 7911-7925 shows that expression of B. amyloliquefaciens subtilisin BPN' in B. subtilis is possible with its own transcription signals, Jacobs et al., Nucleic Acids Res. 13 (1985) 8913-8926 shows that such a strategy is not generally applicable. It 35 was demonstrated that the 5' region of subtilisin Carlsberg derived from B. licheniformis did not contain functional signals for transcription in B. subtilis. To achieve

considerable production it was needed to insert a B. subtilis promoter in front of the gene in question. It is also suggested that not only transcriptional but also translational, secretion or maturation processes may occur
5 less efficient in heterologous hosts, due to a not perfect Shine Dalgarno sequence or even incompatibility of the gene product with the heterologous host.

The problems mentioned above can be avoided when the product is synthesized in a homologous Bacillus strain, of
10 which high production capabilities are proven, with high efficiency resulting in high production levels.

SUMMARY OF THE INVENTION

15 Transformed alkalophilic Bacillus strains are provided for the efficient production strains of mutant Bacillus proteases. The new strains have lost their ability to express their protease encoding genes originally present.

In a preferred embodiment mutant proteases are
20 produced which show close homology with the proteases originally produced by the corresponding strain.

Preferred strains are Bacillus novo species PB92, derivatives thereof and closely related strains, transformed by a mutated protease encoding gene. Said gene shows at
25 least 30%, preferably at least 50% and more preferably at least 80% homology with the wild-type gene of Bacillus PB92 encoding serine protease. The gene is preferably derived from a wild-type gene of an alkalophilic Bacillus strain.

Also provided are methods of constructing the new
30 transformed strains, vectors used thereby and a method of producing mutant proteases.

BRIEF DESCRIPTION OF THE DRAWINGS

35 Figure 1 shows the construction of plasmid pM58 Δ containing parts of the 5' and 3' flanking regions of the alkaline protease gene from Bacillus novo sp. PB92.

Figure 2 schematically shows the introduction of the temperature-sensitive origin of replication from plasmid pE194neo-1 into plasmid pM58Δ, giving the plasmid pEM58Δ.

5 Figure 3 shows the introduction by homologous recombination, of the plasmid pEM58Δ into the 5' flanking region of the protease gene of Bacillus strain PBT110.

10 Figure 4 shows a simplified restriction map of the regions surrounding the deleted protease gene after illegitimate recombination. Two strains were obtained, PBT125 and PBT126.

Figure 5 nucleotide sequence of the HindIII fragments of PBT125 (A) and PBT126 (B).

Figure 6 schematic representation of the introduction of the protease gene into M13 mp18.

15 Figure 7 shows the nucleotide sequence of plasmid pBHA-1.

Figure 8 shows the introduction of the protease gene of Bacillus strain PBT110 in plasmid pBHA1.

20 Figure 9 shows the reorientation of the F1 ori containing BamHI fragment of pBHA1-MXL giving plasmid pBHAR-MXL.

Figure 10 shows the subcloning of the 3' sequence of the high alkaline protease into plasmid pBHAR-MXL, giving plasmid pBHARB-MXL.

25 Figure 11 shows the deletion of the E. coli sequence from plasmid pBHARB-MXL M216Q giving plasmid pBHB-MXL M216Q.

Figure 12 shows the reorientation of the neomycin resistance gene in plasmid pE194neo-1 giving plasmid pE194neo-3.

30 Figure 13 schematically shows the introduction of the temperature-sensitive origin of replication from plasmid pE194neo-3 into plasmid pBHB-MXL M216Q giving plasmid pEN₃Q.

Figure 14 schematically shows the construction of strain Bacillus strain PEP111.

35 Figure 15 schematically shows the construction of strain Bacillus strain PEP211.

DETAILED DESCRIPTION OF THE INVENTION

For the production of alkaline proteases preferably alkalophilic Bacillus strains are used as host cells. For 5 large scale production industrial strains are a need. They originate from organisms which may be isolated from the soil or are available from depositories or other sources and are obtained by genetic modification of such Bacillus strains. Industrial Bacillus strains are defined in EP-A-10 134048. They are characterized by being resistant to genetic exchange, such as phage infection or transformation. The strains are stable and may or may not be capable of spore formation. They are usually prototrophic and modified to provide for high yields of endogenous protein products, such 15 as the enzymes alpha-amylase and various proteases. The yield of an endogenous protein product obtained in an industrial production process can amount to at least 5 g/l (0.5% w/v). Industrial strains also secrete DNases, which result in degradation of DNA in the medium, providing for 20 protection against genetic exchange.

An additional advantage of using alkalophilic Bacilli as production strains is the minimal risk of contamination with other microorganisms, because of the alkaline pH during fermentation.

According to the present invention a method is disclosed for the deletion of a Bacillus protease encoding gene from the genome in such a way that reversion becomes impossible. Said deletion may be a partial deletion but more preferably the gene is completely deleted. The protease 25 negative strain obtained by this method is used for the production of homologous mutant proteases after introduction of an expression vector carrying the gene encoding this mutant protease. It is, however, understood that such a protease negative strain will also be advantageous for the 30 expression of other proteins.

It has been found that by using an industrial protease producing Bacillus strain as a host for the

production of the mutants of this protease, the high efficiency of the production of the original protease is transferred to the mutant protease. This results in a surprisingly superior production efficiency compared to 5 laboratory strains. One chromosomal copy of the mutated gene gives upon expression and secretion in an industrial strain a yield even higher than 50 plasmid copies containing the mutated gene, in a laboratory strain. Moreover, as for the 10 industrial strain a production process including fermentation, recovery and formulation protocols is available the invention that industrial strains are particularly useful for mutant protease production has the advantage of not having to develop a production process as would be the case if a new production host strain was 15 chosen.

It is an aspect of this invention that mutant proteases can be produced efficiently by a homologous Bacillus strain by way of exchanging the chromosomal gene or a part thereof with the corresponding mutated gene. In this 20 way the production capacity for the wild-type protease is eliminated while at the same time introducing production capacity for a mutant protease.

As a host cell for the expression of mutated genes encoding modified or so-called protein engineered proteins 25 it is preferable to use cells in which the genes are structurally expressed at high level.

The present invention also shows (Table 1) that to get a sufficient production of the high alkaline protease derived from Bacillus PB92 in B. subtilis, it was needed to 30 insert a promoter in front of the gene which is active in B. subtilis. If enzymes derived from other Bacilli are produced in B. subtilis insertion of a promoter active in B. subtilis or other expression signals may be necessary, which requires an extra step.

35 High alkaline protease producing bacilli are taxonomically not well classified and are generally referred to as alkalophilic Bacillus strains. For the present

invention we define alkalophilic bacilli as Bacillus strains that grow well under alkaline conditions pH 9-11 (Horikoshi, K. and T. Akiba, 1982, Alkalophilic microorganisms, Springer Verlag, New York). The alkaline 5 proteases produced by such bacilli are also called high alkaline proteases. Examples of Bacillus strains capable of growing at alkaline pH are described in, for example, U.S. Patent Nos. 3,723,250, Re. 30,602 and 4,480,037. An example of an alkalophilic Bacillus host strain is Bacillus novo 10 species PB92 disclosed inter alia in U.S. Patent No. Re. 30,602. Derivatives of these alkalophilic Bacillus strains, that have been optimized for protease production are employed to produce their proteases on industrial scale (See EP-A-0284126). The products are used in several industrial 15 applications e.g. as an additive in laundry detergents. Examples of such products are Maxacal^R (Gist-brocades/IBIS), Savinase^R (NOVO), Esperase^R (NOVO). Mutants of such products have been described in WO 89/06279, where it is said that they are derived from Bacillus lentinus strains, and in the 20 not prepublished European Patent Application EP-A-0328229.

According to a preferred embodiment of the present invention transformed Bacillus strains are provided which produce the mutants described in EP-A-0328229 and WO 89/06279, preferably on an industrial scale.

25 According to the present invention a Bacillus strain is suitably used, in particular an alkalophilic Bacillus, preferably PB92. Another preferred group of Bacillus strains are the asporogenous mutants, of which PBT110 and its derivatives are most preferred. Transformation of 30 alkalophilic Bacillus strains will preferably involve the use of protoplasts from said strains. However, the conventional protoplast transformation protocol as described by Chang, S. and S.M. Cohen, Molec. Genet. 168 (1979) 111-115, does not work for alkalophilic Bacillus strains. 35 The protocols needed for these strains have been disclosed in EP-A-0283075, which is herein included by reference.

Expression of mutant protease genes in homologous hosts necessitates the replacement and/or inactivation of the wild type gene. Some methods can be used.

A) One method is to clone the gene or part thereof, 5 modify it by site-directed mutagenesis and reintroduce the (partial) gene into the cell on a plasmid. By homologous recombination the gene may be introduced into the chromosome. Resulting in a situation in which a wild-type and the mutant gene are tandemly located. After a second 10 recombination the modified sequence is left in the chromosome having thereby effectively introduced the mutation into the chromosomal gene (as described in Figure 14).

B) In another method the chromosomal gene copy is 15 inactivated by insertion or deletion. If insertional inactivation is chosen as a strategy the DNA fragment used as an insert may be any fragment as long as the formation of a complete protein product from the inactivated gene is prevented. Preferably the fragment contains a selectable 20 marker. If deletion of the gene is chosen as a strategy the chromosomal gene fragment that is deleted can be the whole coding region or a part thereof. After the inactivation a mutant copy of the inactivated gene is brought into the cell on a cloning vector.

C) In another method the chromosomal gene copy is 25 mutagenized. In principle it is possible to obtain (specific) mutations *in vivo*, after transformation of bacteria with oligonucleotides which are mutagenic. Such a method has been successfully used in Yeast as described by 30 Moerschell *et al.* Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 524-528. If such a method is performed on a homologous preferably a homologous alkalophilic Bacillus strains this is embodiment of this invention.

The present invention relates to Bacillus strains 35 that are known to be efficient protease producers. In one of the preferred methods the complete alkaline protease coding region is deleted from the vector in vitro, leaving behind

the 5' and 3' flanking sequences. The vector used is preferably a plasmid. To make selection possible a selectable marker is introduced into the plasmid preferably an antibiotic resistance e.g. neomycin resistance.

5 Preferably the vector can selectively be integrated into the chromosome this can be achieved by introducing an inducible origin of replication e.g. a temperature sensitive origin like the one from pE194. Said plasmid is introduced into a homologous host cell. Under high temperature condition, the

10 plasmid is not able to replicate so that chromosomal integrants can be selected for. Integrants are selected at high temperatures in the presence of the relevant antibiotic (e.g. neomycin). Resolution of the plasmid from the host chromosome can leave the flanking regions in the

15 chromosome while removing the coding region. Finally, the resulting mutant selected for has lost the selectable marker gene (is neomycin sensitive) and is protease negative. The final chromosomal configuration is determined by restriction analysis and Southern blotting. WO 88/06623 extensively

20 describes the possible integration mechanisms.

The protease gene, modified by site-directed mutagenesis is now introduced into the protease negative cell on a suitable expression vector. Care is taken that the plasmid carries short or preferably no sequences homologous

25 to the chromosome to avoid plasmid instability.

In another preferred embodiment the flanking regions are left in the plasmid so that the whole of the mutated gene can become integrated in the chromosome. This is especially advantageous if the plasmid turns out to be

30 unstable. The present invention also shows that the amount of recombinant mutant or wild-type protease obtained is comparable or even higher if one copy of the gene is integrated in the genome than if there are several copies of the gene encoded by a plasmid.

35 In yet another embodiment the chromosomal high alkaline protease gene is replaced by the mutant gene by

homologous recombination, without prior isolation of a protease negative strain.

The protease is expressed and can be isolated either from the cells or from the medium when the protein is 5 secreted.

Plasmid pM58, constructed as described in EP-A-0284126, contains the alkaline protease gene. To obtain the inactivation plasmid the coding region of the protease gene is deleted by *Bam*H/*Hpa*I double digestion. The temperature-10 sensitive origin of replication from pE194neo-1 is subsequently introduced. The construct pEM58 is introduced into Bacillus strain PBT110.

Integration by the so-called Campbell-type mechanism took place in the 5' flanking region of the protease gene, 15 resulting in a protease positive strain carrying the entire plasmid vector in its chromosome in the protease locus (as depicted in Figure 3).

Subsequent selection for protease negative derivatives of this strain revealed two strains PBT125 and 20 PBT126, which had lost the protease gene and the neomycin marker. Further analysis showed that in this second step illegitimate recombination had occurred.

Since the obtained Bacillus strains did not show a detectable protease activity they could be used for 25 expression of altered protease genes. It will be clear that PBT125 and PBT126 are only examples. Since illegitimate recombination will give different results it will be essential to determine whether the complete gene has been deleted. To obtain more specific strains, homologous 30 recombinants could have been made.

The examples given here mainly describe two specific embodiments of the invention. One is the production of plasmid encoded mutant protease from a protease negative strain. The other is the production of mutant protease 35 whereby the mutated gene has been introduced in the genome by gene exchange. It will immediately be clear to someone skilled in the art that other embodiments are possible, for

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example; chromosomal integration in a protease negative strain, a mutated gene copy both on a plasmid and integrated into the chromosome, more than one copy of the mutated gene tandemly located or at different positions in the 5 chromosome.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL SECTION

EXAMPLE 1

5

Construction of Inactivation Plasmid pEM58 Δ

Plasmid pM58 (see EP-A-0284126) was digested with restriction enzymes BalI and HpaI. The large fragment containing part of the flanking sequences of the protease gene was purified (Maniatis, Molecular cloning: A Laboratory Manual, Cold Spring Harbor 1982) and ligated. The ligation mix was transformed (Spizizen *et al.*, J. Bacteriol. 81 (1961) 741-746) to Bacillus subtilis DB104 (Kawamura and Doi, J. Bacteriol. 160 (1984) 442-444) and selected for neomycin resistant and protease negative transformants on minimal plates containing 0,4% casein and 20 µg/ml neomycin.

Plasmid pM58 Δ was isolated and characterized by restriction enzyme analysis. As shown in Figure 1 plasmid pM58 Δ contains the flanking sequences of the alkaline protease gene, the gene coding for neomycin resistance and a Bacillus origin of replication.

To construct an integration plasmid containing a temperature sensitive origin of replication, pM58 Δ was digested with restriction enzymes XbaI and BglII. The fragment containing the alkaline protease flanking sequences was purified and ligated to the (purified) XbaI/BglII fragment of pE194neo-1 (EP-A-0284126) containing the temperature sensitive origin of replication, derived from pE194 (Jordanescu *et al.*, Plasmid 1 (1978) 468-479) after said fragment was purified as formerly described. The ligation mix was transformed to B. subtilis DB104 and selected for neomycin resistance (see above). Plasmid pEM58 Δ was isolated and characterized (Figure 2). It contains the neomycin resistance gene, the temperature sensitive origin of replication from pE194 and the flanking sequences of the alkaline protease gene.

This plasmid pEM58 Δ was used to transform Bacillus strain PBT110.

EXAMPLE 2

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Construction of a Protease Negative
Alkalophilic Bacillus Strain

10 A. Protoplast transformation of Bacillus PBT110 by plasmid
pEM58 Δ

15 Bacillus strain PBT110 is an asporogenous mutant of Bacillus novo species PB92 (U.S. Patent No. Re. 30,602) and was obtained by classical (UV) mutation procedures. This strain was transformed with plasmid pEM58 Δ similar to the method described in EP-A-0284126. Prior to integration experiments, transformants were checked by restriction enzyme analysis whether they contained the relevant plasmid.

20 B. Integration of pEM58 Δ in Bacillus PBT110 chromosome

Integration of pEM58 Δ in Bacillus strain PBT110 chromosome was performed as described in EP-A-0284126. Selection for integrants occurred at a neomycin concentration of 1 μ g/ml. The genetic organization of the integrant was determined by restriction enzyme analysis followed by Southern blotting and hybridization analysis and is shown in Figure 3. It appeared that integration of pEM58 Δ took place through a so-called Campbell-type mechanism by 30 homologous recombination in the 5' flanking region of the alkaline protease gene, resulting in Bacillus strain PBT110-INT5.

C. Selection for protease negative strains

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Bacillus strain PBT110-INT5 was inoculated in 100 ml

tryptic soya broth (TSB) containing 1 µg/ml neomycin and incubated for 24 hours at 50°C.

After 24 hours 0.1 ml of the culture obtained was inoculated in a 500 ml shake flask containing 100 ml PBT minimal medium: K₂HPO₄, 17.42 g/l; glutamate, 13.36 g/l; citrate·H₂O 2 g/l; FeSO₄·7H₂O, 0.05 g/l; ZnSO₄·7H₂O, 1 mg/l; MnSO₄·H₂O, 1 mg/l; CaCl₂·2H₂O, 10 mg/l; MgSO₄·7H₂O, 0.2 g/l; CuSO₄·5H₂O, 0.5 mg/l; H₃BO₃, 0.5 mg/l; Na₂MoO₄·2H₂O, 0.5 mg/l; CoCl₂·6H₂O, 0.5 mg/l; Biotine, 0.5 mg/l; saccharose, 20 g/l; (pH 8.0, sterilized for 20 min at 120°C). After sterilization 1 mg/l thiamine was added.

This culture was incubated for 4 days at 37°C. After 4 days, 1 ml of culture was diluted in 100 ml of the same medium and incubated for 4 days. After 4 days the culture was plated on PBT minimal medium plates containing additionally 0.4% casein and 15 g/l agar. Colonies which lacked a detectable protease halo were considered protease minus and were checked on the absence of the gene encoding the PBT110 alkaline protease.

20

D. Characterization of protease negative strains

Strains which did not show a detectable halo on the casein plates described in the previous section were primarily tested on the presence of a neomycin sensitive and asporogenous phenotype. Two strains, Bacillus PBT125 and PBT126, which contained the protease negative and asporogenous phenotype were tested for protease production in 500 ml shake flasks containing 100 ml protease production medium, as described in U.S. Patent No. Re. 30,602. Neither strain produced detectable amounts of protease.

The genetic organization of the two strains was determined by restriction enzyme analysis and chromosomal blotting and is shown in Figure 4. No homologous but illegitimate recombination had occurred, resulting in two strains with completely deleted protease and neomycin resistance genes. However, still a small plasmid fragment of

undefined size appeared to be left in the chromosome after deletion of the protease gene. The sequence of this fragment was determined as follows. The chromosomal HindIII fragments of strains PBT125 and PBT126 containing the plasmid/5 chromosome junctions were ligated in phage vector M13 mp18 (Messing et al., Nucl. Acids Res. 9 (1981) 303-321) and transfected to E. coli JM101 according to the procedure described by Cohen et al., Proc. Natl. Acad. Sci. USA 69 (1972) 2110-2114. After phage propagation in E. coli JM101,10 ssDNA was isolated (Heidecker et al., Gene 10 (1980) 69-73).

The inserts were sequenced using the method described by Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977) 6463. The results are shown in Figures 5A and 5B, respectively.

15

EXAMPLE 3Construction of Protease Production and Mutation Vectors20 A. Subcloning of the PB92 protease gene in phage M13 mp18

Plasmid pM58 was digested with HpaI and BalI. After purification of the DNA fragment containing the protease gene (Maniatis, 1982) this fragment was ligated into phage 25 M13 mp18 which was digested with SmaI. The ligation mixture was transfected to E. coli JM101 (Cohen et al., supra). After phage propagation in E. coli JM101, dsDNA was isolated according to the method described by Birnboim and Doly (Nucl. Acids Res. 7 (1979) 1513-1523). The insert and its 30 orientation were checked by restriction enzyme analysis. Vector mp18-MXL was used for further subcloning experiments and is shown in Figure 6.

B. Subcloning of the PB92 protease gene in plasmid pBHA1

35

Figure 7 shows the nucleotide sequence of plasmid pBHA1. This plasmid is derived from the twin vector system

pMa/c5-8, described by Stanssens et al. (Nucleic Acids Res. 17 (1989) 4441-4454) in which an additional promoter was inserted. Plasmid BHA1 consists of the following fragments:

- 5 pos 11-105: bacteriophage FD, terminator;
pos 121-215: bacteriophage FD, terminator;
pos 221-307: a part of plasmid pBR322 (viz. positions 2069-2153);
pos 313-768: bacteriophage F1, origin of replication (viz.
10 pos 5482-5943);
pos 772-2571: part of plasmid pBR322, viz. the origin of
replication and the β -lactamase gene;
pos 2572-2685: transposon Tn903, complete genome;
pos 2719-2772: tryptophan terminator (double);
15 pos 2773-3729: transposon Tn9, the chloramphenicol-acetyl-
transferase (=cat) gene. The nucleotides at
pos 3005(A), 3038(C), 3302(A) and 3409(A)
differ from the wild-type cat coding
sequence. The mutations were introduced to
20 eliminate the NcoI, BalI, EcoRI and PvuII
sites;
pos 3730-3804: multiple cloning site;
pos 3807-7264: part of plasmid pUB110 (viz. the replication
function and kanamycin resistance gene,
25 EcoRI-PvuII fragment) (McKenzie et al.,
Plasmid 15 (1986) 93-103 and Plasmid 17
(1987) 83-85);
pos 7267-7331: multi cloning site.
- 30 The fragments were combined using known cloning
techniques, e.g., filling in sticky ends with Klenow,
adapter cloning, etc. All data were derived from Genbank^R
National Nucleic Acid Sequence Data Bank, NIH, U.S.A.
Plasmid pMC5-8 was deposited under DSM 4566.

pBHA/C-1 was modified to obtain the twin vector system pBHARB-MXL/pBHCRB-MXL, as follows:

1. Vector mp18-MXL was digested with KpnI and HincII. The DNA fragment containing the protease gene was purified 5 and ligated into pBHA1 which was digested with EcoRV and KpnI. The ligation mixture was transformed to E. coli JM101 (Maniatis, 1982) and plated on LC⁺ plates containing 10 g/l tryptone, 5 g/l Yeast extract (Difco), 8 g/l NaCl, 25 g/l thymine, 1 g/l MgSO₄·7H₂O, 100 µg/ml ampicillin and 20 10 µg/ml neomycin pH 7.0. Plasmid DNA of transformants was isolated (Birnboim, supra) and characterized by restriction enzyme analysis. In this way pBHA1-MXL was isolated (see Figure 8).

2. In order to sequence mutations introduced in the 15 protease gene with an available set of oligonucleotides, it was necessary to reverse the orientation of the F1 origin compared to the protease gene. This was performed in the following way: plasmid pBHA1-MXL was digested with BamHI and religated. The ligation mix was transformed to E. coli WK6 20 (Maniatis, supra) and selected for neomycin or ampicillin resistance on LC⁺ plates containing 10 g/l tryptone, 5 g/l Yeast Extract (Difco), 8 g/l NaCl, 25 mg/l thymine, 1 g/l MgSO₄·7H₂O, 100 µg/ml ampicillin and 20 µg/ml neomycin pH 7.0. Plasmid DNA of transformants was isolated (Birnboim, 25 supra) and characterized by restriction enzyme analysis. In this way pBHAR-MXL was isolated, which differs from pBHA-MXL in that the orientation of the E. coli sequence is reversed, (see Figure 9).

3. For the introduction of additional flanking 30 sequences in plasmid pBHAR-MXL, the following digestions were made. Plasmid pM58 was digested with SphI and HindIII. Plasmid pBHAR-MXL (Figure 10) was digested with SphI and HindIII. The larger fragment was purified. Both digests were ligated and transformed to B. subtilis DB104 and selected 35 for neomycin resistance and protease activity on minimal plates containing 10 µg/ml neomycin and 0.4 % casein. Transformants were characterized by restriction enzyme

analysis. One of these, pBHARB-MXL (Figure 10), was used for further experiments.

C. Mutagenesis of the PB92 gene in plasmid pBHARB-MXL

5

Mutagenesis was carried out with the twin vector system pBHARB-MXL/pBHC RB-MXL (Stanssens, *supra*).

A method based on the gapped-duplex approach (Kramer *et al.*, Nucl. Acids Res. 12 (1984) 9441) and a phasmid (phage/plasmid hybrid) was used. Essentially the method rests on a gapped-duplex DNA intermediate consisting of a gapped strand (- strand) containing a wild-type antibiotic resistance marker and a template strand (+ strand) carrying an amber mutation in the gene conferring resistance to the antibiotic. After annealing, the mutagenic oligonucleotide becomes incorporated in the gapped strand during in vitro gap-filling and sealing reaction. The resultant molecules are used to transform a mismatch repair deficient (Mut S) host in which the linkage between the intended mutation and the antibiotic resistance marker is preserved. The mixed phasmid population, isolated from this strain, is then allowed to segregate in a suppressor negative host strain. Transformants are plated on antibiotic containing medium, thus imposing a selection for progeny derived from the gapped strand.

In the pMa type vector nucleotide 3409 is changed from G to A, while in the pMc type vector nucleotide 2238 is changed from G to C, creating amber stopcodons in the chloramphenicol-acetyltransferase gene and β -lactamase gene, respectively, rendering said genes inactive.

To perform mutagenesis the target DNA fragment is cloned into the multiple cloning site of pMa5-8 or a derivative thereof. A gapped duplex between pMa5-8 containing the target DNA and pMc5-8 is then constructed.

The single strand gap, consisting of the target DNA, may be subjected to mutagenesis with a mutagenic oligo-

nucleotide, with long synthetic oligonucleotides having a low level of misincorporated nucleotides, using chemical or enzymatic misincorporation of nucleotides. For a detailed description, see Ausubel *et al.*, 1987, Current Protocols in Molecular Biology, John Wiley & Sons Inc. New York; or B. Perbal, 1988, A practical Guide to Molecular Cloning, 2nd ed. John Wiley & Sons Inc; New York.

D. Construction of Vectors for Industrial Protease Production

After the introduction of the desired mutation(s) in plasmid pBHARB-MXL, the unwanted *E. coli* sequences from the plasmid were deleted as follows: plasmid pBHARB-MXL containing the relevant mutation was digested with BamHI and religated under diluted conditions. The ligation mixture was transformed to *B. subtilis* DB104 and selected for neomycin resistance and protease activity on minimal plates containing 20 µg/ml neomycin and 0.4 % casein. DNA of transformants was isolated and characterized by restriction enzyme analysis. In this way vectors were isolated lacking *E. coli* DNA, which are suitable for commercial production of proteins.

The foregoing procedure is illustrated in Figure 11 where mutation M216Q is taken as an example, resulting in plasmid pBHB-MXL M216Q. M216Q, and also M216S, S160D and N212D referred to hereinafter, are mutant proteases of *Bacillus* PB92, described in EP-A-0328229.

30

EXAMPLE 4

Protoplast Transformation of Bacillus PBT125
by Plasmid pBHB-MXL Derivatives

35 *Bacillus* strain PBT125 was transformed with plasmid pBHB-MXL or plasmids derived thereof containing mutant protease genes, following the transformation procedure

described in EP-A-0284126. Prior to production studies, transformants were checked by restriction enzyme analysis to contain the relevant plasmid.

5

EXAMPLE 5Construction of Protease Integration VectorsA. Integration vector pEN₃Q

10

Plasmid pE194neo-1 (EP-A-0284126) was digested with SallI and religated. The ligate was transformed to B. subtilis DB104 and selected for neomycin resistance on minimal plates containing 20 µg/ml neomycin. Plasmid pE194 neo-3 was isolated and checked on the presence of the neomycin resistance gene in a reversed orientation (see Figure 12). Plasmid pE194neo-3 was digested with HpaI and BglII. The fragment containing the temperature sensitive origin of replication was purified (Maniatis, supra).

20

Plasmid pBHB-MXL M216Q (see Example 3 and Figure 11) was digested with BglII and BalI. The fragment containing the protease gene was purified. Both fragments were ligated. The ligation mix was transformed to B. subtilis DB104 and selected for protease activity and neomycin resistance as described before. Transformants were characterized by restriction enzyme digestions. One of these, containing plasmid pEN₃Q, was selected (Figure 13). This plasmid contained the neomycin resistance gene, the t.s. origin of replication from plasmid pE194neo-3 and the M216Q mutation in the PB92 protease gene.

B. Integration vector pEN₃S.

In the same way as described for integration vector pEN₃Q, pEN₃S was constructed using pBHARB-MXL M216S as starting vector.

EXAMPLE 6Construction of Bacillus Strains PEP111 and PEP1125 A. Protoplast transformation of Bacillus PBT110 by Plasmid pEN₃Q

Bacillus strain PBT110 was transformed with plasmid pEN₃Q as described in EP-A-0284126. Prior to integration experiments, it was checked by restriction enzyme analysis whether the transformants contained the relevant plasmid.

B. Integration of pEN₃Q in Bacillus PBT110 chromosome

15 Integration experiments with plasmid pEN₃Q in Bacillus strain PBT110 chromosome were performed as described in EP-A-0284126. Selection for integrants occurred at a neomycin concentration of 1 μ g/ml at non-permissive temperatures (50°C) for plasmid replication. To 20 check the integration of pEN₃Q in the chromosome, chromosomal DNA of potential integrants was isolated, digested with HindIII or ClaI, run on a 0.8% DNA agarose gel and blotted to nitrocellulose (Southern, J. Mol. Biol. 98 (1975) 503-517) and hybridized with ³²P labeled nick-translated pEN₃Q DNA (Maniatis, 1982).

It was shown that integration of pEN₃Q had occurred by homologous recombination resulting in a strain (PBT110M/Q) with two protease genes (one wild type and one mutant M216Q) tandemly located on the chromosome. Thus, 30 integration of pEN₃Q occurred by a so-called Campbell-type mechanism as depicted in Figure 14 (see also EP-A-0284126).

C. Selection of neomycin sensitive recombinants

35 Neomycin sensitive recombinants were selected, which contain either the wild-type or the mutant protease gene in the chromosome (hereinafter called "outrecombinants"). The

selection procedure is similar to the procedure described before in Example 2C.

Strain PBT110 M/Q was used as starting strain. After strain PBT 110 M/Q was incubated in PBT minimal medium, the 5 culture was plated on PBT minimal medium plates containing 0.4% casein and 15 g/l agar. These plates were incubated for 48 h at 37°C and replica plated to Heart infusion (HI) plates containing 1 µg/ml neomycin and no neomycin, respectively. Neomycin sensitive colonies were considered 10 outrecombinants.

D. Characterization of neomycin sensitive recombinants.

Chromosomal DNA of potential outrecombinants was 15 isolated, digested with ClaI or HindIII, run on a 0.5% agarose gel, blotted to nitrocellulose (Southern, 1975) and hybridized with ³²P labeled nick translated pEN₃Q (Maniatis, 1982). Since mutation M216Q results in the removal of a ClaI site, the strains containing a wild-type 20 protease or a mutant protease gene, and the intermediate strain PBT110 M/Q can be easily distinguished, see Fig. 14.

An outrecombinant containing the M216Q mutant protease gene of PB92 was isolated. After it was shown that a gene replacement of PB92 wild-type protease to PB92 mutant 25 protease had occurred, the product of the strain was characterized by its biochemical parameters (k_{cat} , K_m), oxidation resistance, specific activity and wash performance, as described EP-A-0328229. All results were equal to a control protease M216Q, indicating that the 30 product of the transformed strain was indeed the PB92 protease containing the mutation M216Q. This transformed strain is called Bacillus PEP111.

Similar experiments were performed using vector pEN₃S to construct Bacillus strain PEP112, which contains 35 the PB92 protease with mutation M216S.

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EXAMPLE 7

Construction of *Bacillus* Strains PEP211 and PEP212

5 *Bacillus* strain PEP111 was transformed with plasmid pEN₃Q, following the procedure described in EP-A-0284126. Prior to the integration procedure, it was checked by restriction enzyme analysis whether the transformants contained the relevant plasmid. The integration procedure 10 and the selection for integrants (at a neomycin concentration of 20 µg/ml at non-permissive temperatures (50°C) for plasmid replication) was performed as described in EP-A-0284126.

15 To check the integration of plasmid pEN₃Q in the chromosome, chromosomal DNA of potential integrants was isolated and digested with HindIII, run on 0.8% agarose gels, blotted to nitrocellulose (Southern *et al.* 1979) and hybridized with ³²P labeled nick translated pEN₃Q. It was shown that a strain containing two mutant (M216Q) PB₉₂ protease genes, separately located on the chromosome was isolated. This strain is called PEP211. Its chromosomal organization is shown in Figure 15.

20 To obtain an analogous strain containing two protease genes with mutation M216S, strain PEP112 containing a mutant gene (M216S) after gene replacement of the wild-type gene, was transformed with plasmid pEN₃S and procedures were followed as described above. These experiments resulted in strain PEP212 containing two separately located PB₉₂ mutant M216S protease genes on the chromosome.

30

EXAMPLE 8

Production of Mutant Proteases

35 Production of protease mutants was performed with transformed *Bacillus* strains in which the mutated protease

encoding gene was either localized on a plasmid or on the chromosome.

For plasmid encoded production 2 types of strains were used, the alkalophilic strain Bacillus PBT125, as herein described, and B. subtilis strain DS12367, an asporogenic strain derived from B. subtilis DB104 (Kawamura et al., J. Bacteriol. (1984) 160, 442-444. These strains were transformed with pBHB-MXL derived plasmids. Bacillus PBT125 and B. subtilis DS12367, which do not possess protease encoding plasmids, were used as control strains.

For production with strains containing chromosomally integrated protease mutant genes the Bacillus PEP strains, as herein described, were used. Bacillus PBT strains 108 and 110, containing the wild-type protease genes were used as control strains. PBT108 is a strain containing two wild-type genes separately located on the chromosome, see EP-A-0284126. For PBT110, see Example 2A.

To check the activity of the original promoter of the PB92 protease gene in B. subtilis, a construct was made, pbHB-MXLR, in which the orientation of the protease gene to the HpaII promoter (Zyprian et al., DNA 5 (1986) 219-225) was reversed, so that the expression of the protease gene is only directed by its original promoter.

The productions were performed in 500 ml shake flasks containing 100 ml protease production medium.

When alkalophilic Bacillus strains were used cultures were incubated, after inoculation, for 40 hours at 37°C in a production medium as described in U.S. Patent No. Re. 30,602. In case of plasmid containing strains, neomycin (20 µg/ml) was added.

When B. subtilis DS12367 strains were used, a comparable medium containing 12.5 g/l Yeast Extract (Difco), 0.97 g/l CaCl₂.6H₂O, 2.25 g/l MgCl₂.6H₂O, 20 mg/l MnSO₄.4H₂O, 1 mg/l CoCl₂.6H₂O, 0.5 g/l citrate, 0.5 ml antifoam 5693, 6% w/w maltose, 0.2 M phosphate buffer pH 6.8 was applied. In case of plasmid containing strains, neomycin (20 µg/ml) was added.

Cultures containing B. subtilis strains were incubated for 65 hours at 37°C. In all cases the shake-flasks were inoculated with 0.1 ml of a Tryptic Soya Broth culture containing 20 µg/ml neomycin containing the relevant 5 strain which had been incubated for 24 hours at 37°C.

Protease activity was assayed using dimethylcasein as substrate as described by Lin et al., J. Biol. Chem. 244 (1969) 789-793. The specific activities of the protease mutants (EP-A-0328229) were used to determine the production 10 on mg basis.

The results of the fermentation experiments are summarized in the following Table 1.

Table 1

	<u>Strain</u>	<u>Relative protease production (%)</u>	<u>Mutation</u>
15	PBT110	100	WT
20	PBT125	0	--
25	PBT108	120	WT
30	PEP111	100	M216Q
35	PEP112	100	M216S
40	PBT125 pBHB-MXL	95-100	WT
	PBT125 pBHB-MXL M216Q	95-100	M216Q
	PBT125 pBHB-MXL M216S	95-100	M216S
	PBT125 pBHB-MXL S160D	95-100	S160D
	PBT125 pBHB-MXL N212D	95-100	N212D
	PEP211	120	M216Q
	PEP212	120	M216S
	DS12367	0-1	--
	DS12367 pBHB-MXL	4	WT
	DS12367 pBHB-MXL	40	WT
	DS12367 pBHB-MXL M216Q	40	M216Q
	DS12367 pBHB-MXL M216S	40	M216S
	DS12367 pBHB-MXL S160D	40	S160D
	DS12367 pBHB-MXL N212D	40	N212D

All publications (including patents and patent applications) mentioned in this specification are 45 indicative to the level of skill of those skilled in the art to which this invention pertains. All publications are

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- 27 -

herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in 5 some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

10

CLAIMS

1. A method for the production of proteases differing in at least one amino acid from the wild-type protease, said 5 method comprising the use of a homologous alkalophilic Bacillus strain as expression host said strain being unable to produce the wild-type protease.
2. A method according to Claim 1, employing as 10 expression host a protease negative alkalophilic Bacillus strain.
3. A method according to Claim 2, employing as expression host a protease negative alkalophilic Bacillus 15 strain, wherein the wild-type protease gene is deleted from the genome.
4. A method according to any one of Claims 1 to 3, employing a protease negative derivative of Bacillus novo 20 species PB92.
5. A method according to any one of Claims 1 to 4, employing an asporogenic alkalophilic Bacillus strain.
- 25 6. A method according to any one of Claims 1 to 5, employing an asporogenic Bacillus from which the wild-type protease gene has been deleted by homologous or illegitimate recombination.
- 30 7. A method according to any one of Claims 2 to 6, wherein the mutant high alkaline protease is plasmid encoded.
- 35 8. A method according to Claim 1, 2 or 3, wherein the wild-type high alkaline protease has essentially the amino acid sequence of Bacillus novo species PB92 protease.

9. A method according to Claim 1, wherein the wild-type alkaline protease gene is deleted from the chromosome, and at least one copy of a mutant high alkaline protease gene is inserted into the genome.

5

10. A method according to Claim 9, wherein at least one copy of a mutant high alkaline protease gene is inserted into the genome and another copy is plasmid encoded.

10 11. A method of obtaining an alkalophilic Bacillus strain having a reduced extracellular alkaline protease level comprising:

- 15 - isolating a gene, including the 5' and/or 3' noncoding regions thereof, coding for alkaline protease,
- 20 - inserting the gene into a cloning vector,
- 25 - deleting the coding region of the gene from the cloning vector,
- transforming a Bacillus strain with the cloning vector,
- growing the transformants under conditions in which the replication function of the vector is inactive,
- selecting transformants which have lost the DNA sequence encoding the alkaline protease.

25

12. A method according to Claim 11, wherein said alkalophilic Bacillus strain is Bacillus novo species PB92 or a derivative thereof.

30 13. An alkalophilic Bacillus strain obtained according to Claim 11, transformed with a plasmid expression vector containing a mutant high alkaline protease gene.

35 14. A Bacillus strain according to Claim 13, wherein the alkalophilic Bacillus strain is a derivative of Bacillus novo species PB92, obtained by homologous or illegitimate recombination.

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- 31 -

15. An alkaline protease differing in at least one amino acid from the wild-type protease produced by a Bacillus strain having at least one copy of the mutated gene and no copy of the wild-type gene giving an active protease.

5

16. An alkaline protease according to Claim 15, produced by an alkalophilic Bacillus strain.

17. An alkaline protease according to Claim 15 or 16,
10 where the mutant gene is provided on a plasmid and/or in the genome.

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- 32 -

EFFICIENT PRODUCTION OF MUTANT PROTEASES

5

Abstract of the Disclosure

Alkalophilic Bacillus strains are provided which efficiently produce mutant Bacillus proteases and which are incapable of expressing the wild-type proteases.

8

Figure 1

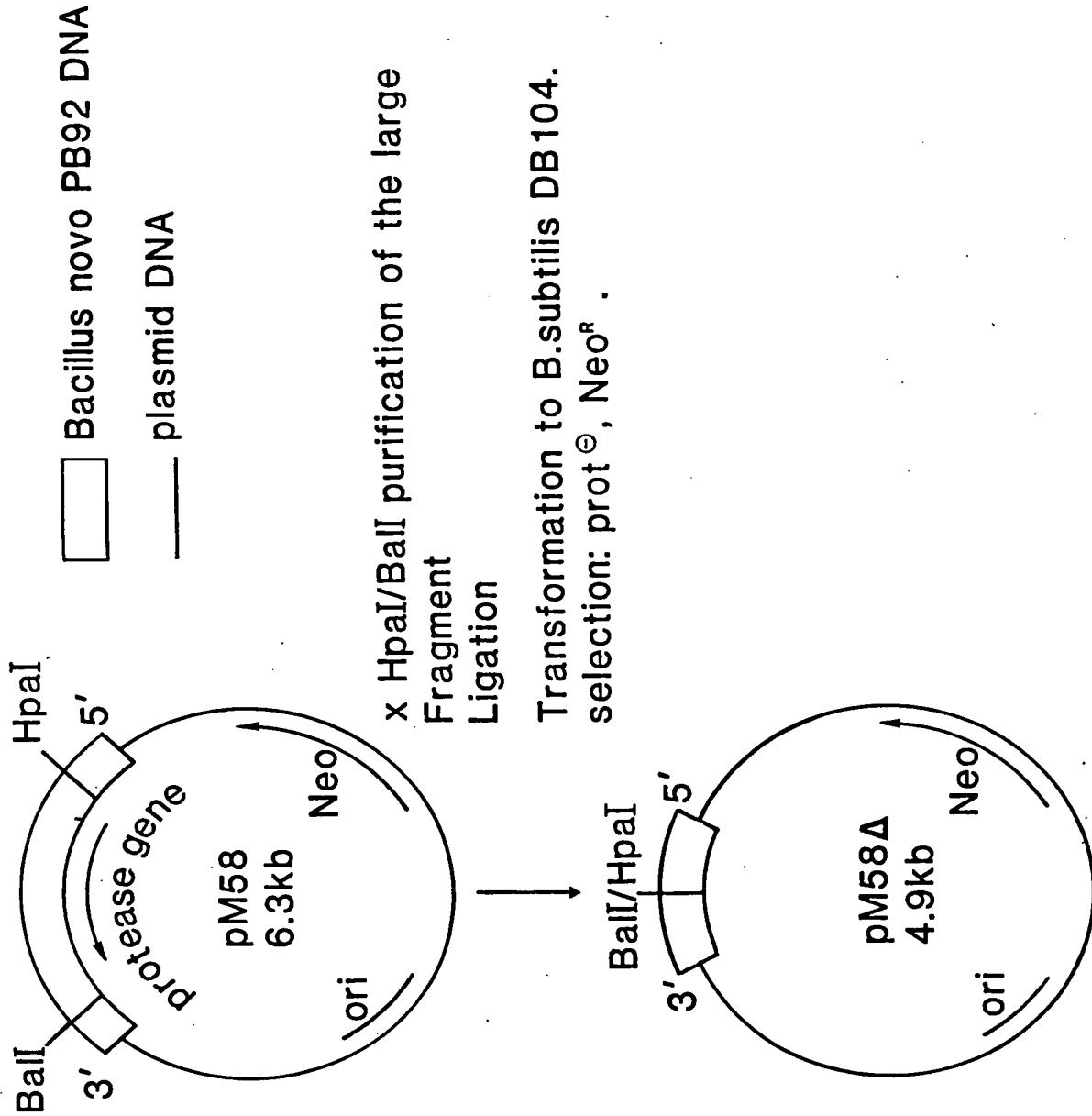
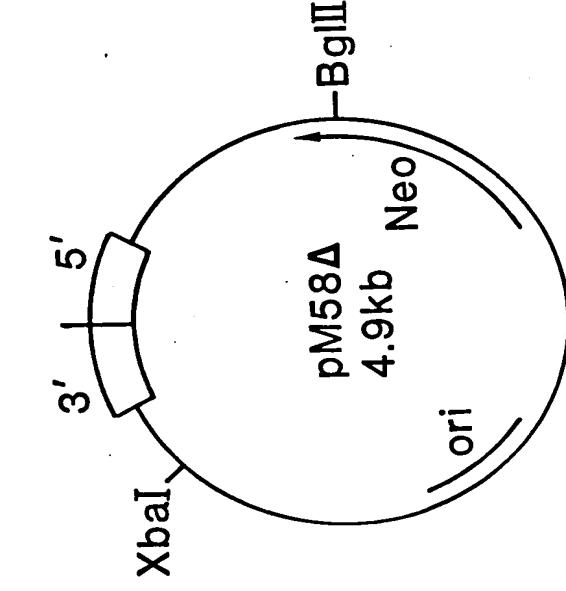


Figure 2

\times XbaI/BglII purification of the
Bacillus novo DNA containing fragment

\times XbaI/BglII purification of the DNA
fragment containing the T.S.ori.

Ligation
Transformation to B. subtilis DB 104
Selection : Neo^R.

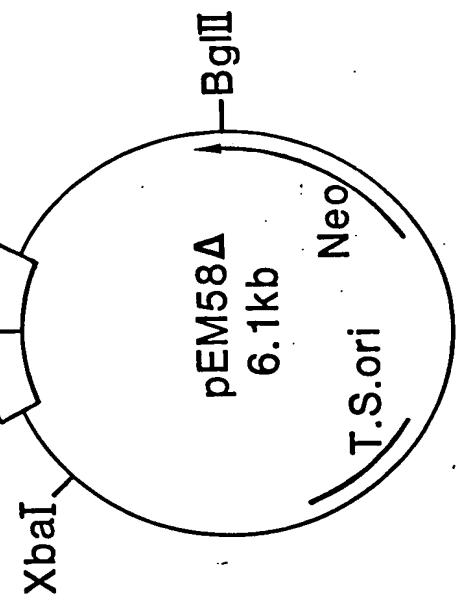


Figure 3 EcoRI 4.5kb EcoRI 2.5kb EcoRI chrom.DNA PBT110

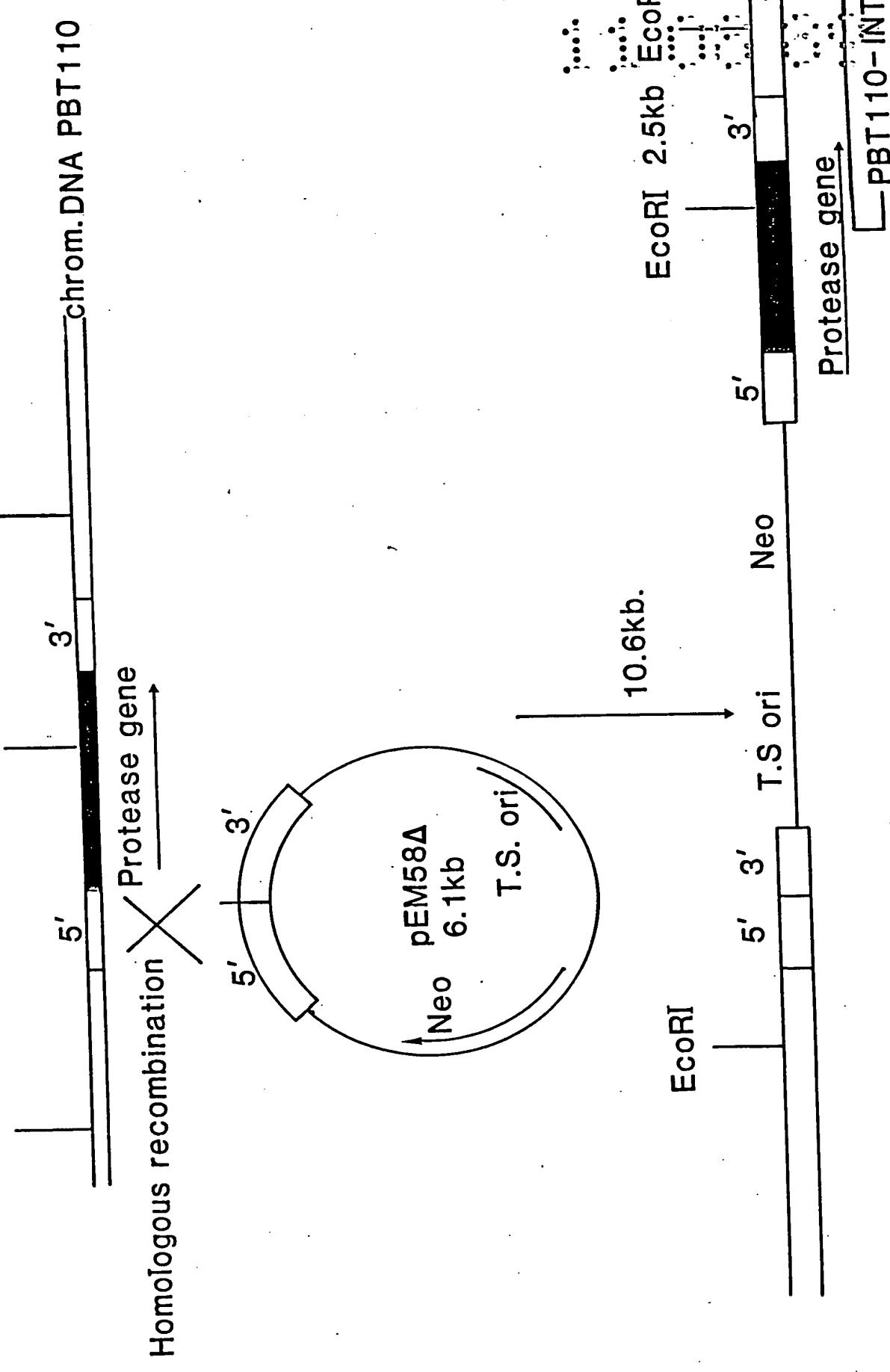
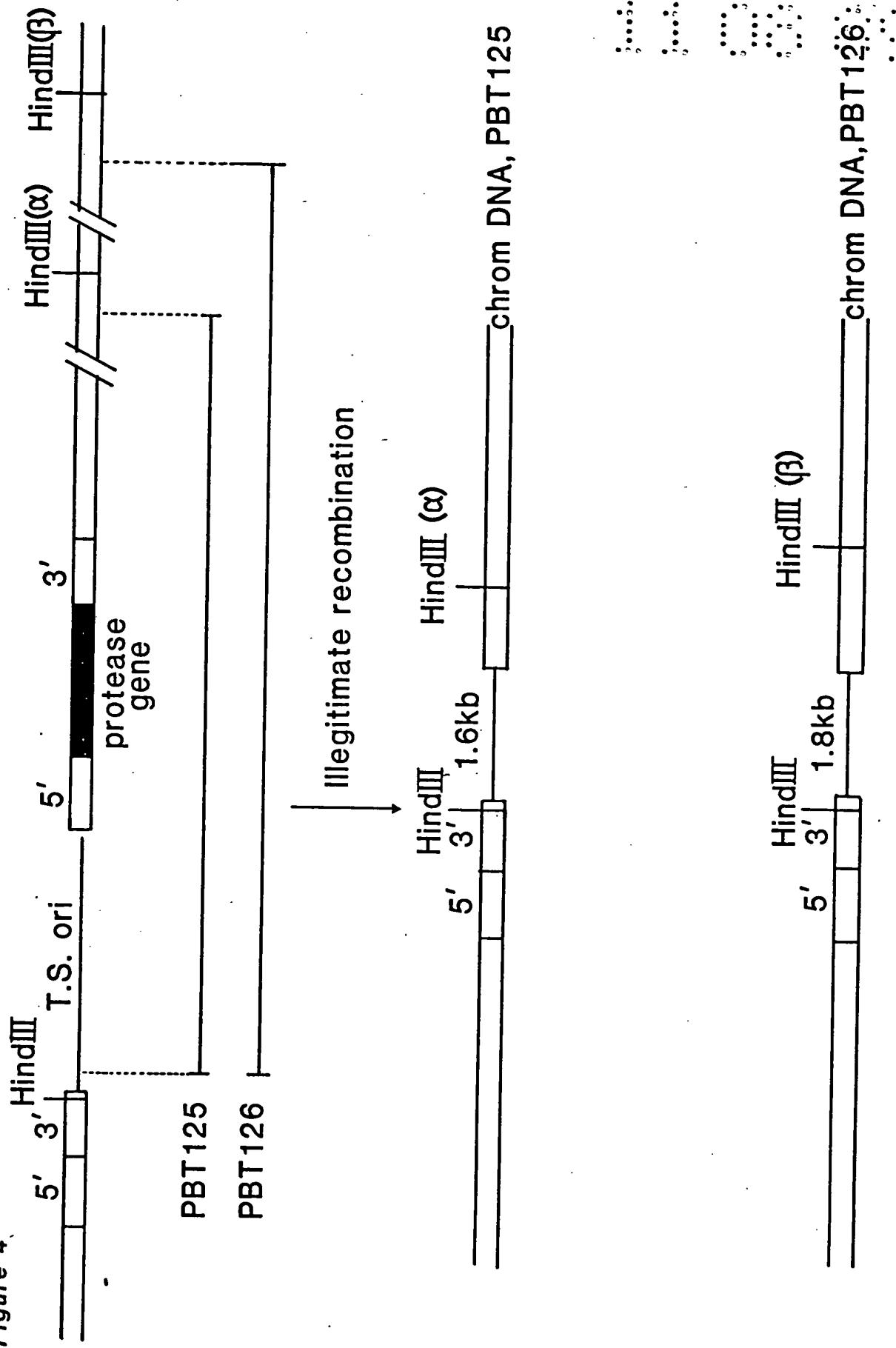


Figure 4

HindIII 40 80
AAGCTTTCAAATGATCCAGGAGAACAAAACGATTGGAGGAAAGTTATAAATTATTTCCGAAACGATATGGCAAGC

AAAATATTGCTTATGCAACAGTCATAATGATGAGCAAACCCCTCACATGCATTAGGTGTTGCCATGGCGATGGAA 120 160

AAACTGCAAGGAAAAATGTGTTAACGTCAGAACTGTTATGGCTACAAGATAAATTCCCCGAGCATATGAAAAAACA 200 240

GGGTTTGAGTTGAAGCGTGGTAACGTGGCTCTGACCGTAAACATATTGAGACAGCTAAATTAAAAACAAACTTTGG 280 320

XbaI 360 400
AAAAAGAGATTGATTCTAGAAAATGAATACGGTAAGGAAATATGCTGTATGCGACTGTCCATCTGGATGAAAGAGTC

CCACATATGCACTTGGTTGTCCCTTAACAGAGGACGGGAGATTGTCTGCAAAGAACAGTTAGGCAACAAGAAAGA 440 480

CTTTACTCAATTACAAGATAGATTAAATGAGTATGTGAATGAGAAAGGTTATGAACTTGAAAGAGGCACGTCAAAGAGG 520 560

TTACAGAACGAGAACATAAGCGATGGATCAGTACAAGAAAGATACTGTATTTCATAAACAGGAACGTGCAAGAAGTTAAAG 600 640

GATGAGTTACAGAACGAAATAAGCAGTTACAGAGTGGAAATAGACCATACTGAGGTCTACGAAACCTTTGATTATGAAAA 680 720

TGAGCGTACAGGTTGTTCTGGACGTGAAGAGACTGGTAGAAAGATATTAACGCTGATGAATTGAACGCCCTGCAAG 760 800

PstI 840 880
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TTAAAAAAACGTAGAGAGAGTTGAAAGAAGTAGTGAATACATGAAAGAGGGTATCACGAAAAAGTAAAGAGGTAA 920 960

TAAATTAAAGCGAGAGAATGATAGTTGAATGAGCAGTTGAATGTATCAGAGAAATTCAAGCTAGTACAGTGACTTTAT 1000 1040

ATCGTGCTGCGAGGGCGAATTCCCTGGGTTGAGAAAGGGTTAATAGGCTAAAGAGAAATTCTTAATGATTCCAAA 1080 1120

TTTGAGCGTGTGGACAGTTATGGATGTTGACAGGATAATGTCCAGAACGGTCGATAGAAAGCGTGAGAACAGCGTAC 1160 1200

AGACGATTTAGAGATGTAGAGGTACTTTATGCCGAGAAAACCTTTGCGTGTGACAGTCCTAAATACTTAGAGCG 1240 1280

TAAGCGAAACTAGTAGCGACAGCTATTAACTTTCGGTTGCAAAGCTCTAGGATTTAAATGGACGCAGCGCATCACACGC 1320 1360

AAAAAGGAAATTGGAATAATGCGAAATTGAGATGTTAATTAAAGACCTTTGAGGTCTTTCTTAGATTTTG 1400 1440
 plasm.seq. chrom.seq.

GGTTATTAGGGAGAAACATAGGGGGTACTACGACCAAAATGGAGGAATACGGCAGCGCGAGCATGGTGGATGCTTG 1520

AATTCTAGGCAACAACACGCCCTATTGACGGTGGGATTCAAGGCTTGGAAAGAACAGTGGCTACCCGACAACCGGGA 1560 1600

HindIII
TGAGGTGAAGCTT

figure 5A

HindIII 40 80
AAGCTTTCAAATGATCCAGGAGAACAAAACGATTGGAGGAAAGTATAAATTATTTCCCACGATAATGGGAAGC

120 160
 AAAATATTGCTTATGCAACAGTTCATATGAGCAAACCCCTCACATGCATTTAGGTGTGCCTATGCGTGATGGA

200 240
 AAACTGCAAGGAAAAATGTGTTAACGTCAAGAACTGTTATGGCTACAAGATAAATTCCCCGAGCATAATGAAAAAAC

280 320
 GGGTTTGAGTTGAAGCGTGGTGAACGTGGCTCTGACCGTAAACATATTGAGACAGCTAAATTAAAAACAAACTTGG

XbaI 360 400
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440 480
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520 560
 CTTTACTCAATTACAAGATAGATTAATGAGTATGTGAATGAGAAAGGTATGAACCTGAAAGAGGCACGTCCAAGAGG

600 640
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680 720
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760 800
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PstI 840 880
 AAACAATCTCTTGCAGAACGGATTGTTGATGATTACGAAAATATTAAGAGCACAGACTATTACACAGAAAATCAAGAA

920 960
 TTAACAAAACGTAGAGAGAGTTGAAAGAAGTAGTAGTGAATACATGAAAGAGGGTATCACGAAAAAGTAAAGAGGTTAA

1000 1040
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1080 1120
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1160 1200
 TTTGAGCGTGTGGACAGTTATGGATGTTGATCAGGATAATGTCCAGAACGGTCGATAGAAAGCGTGAGAACAGCGTAC

1240 1280
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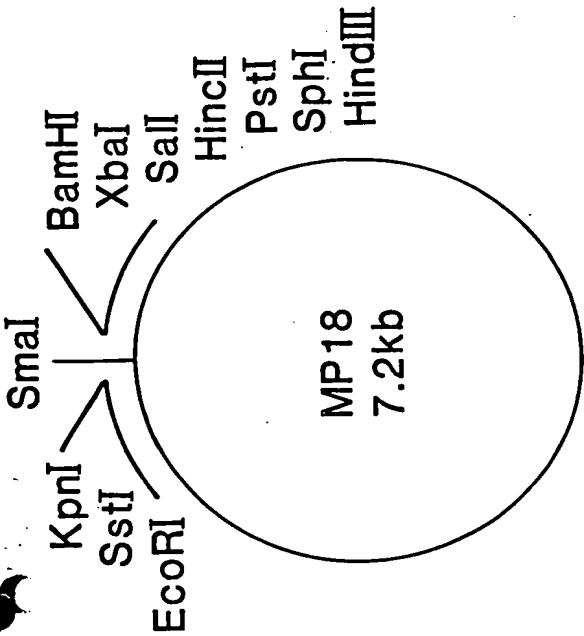
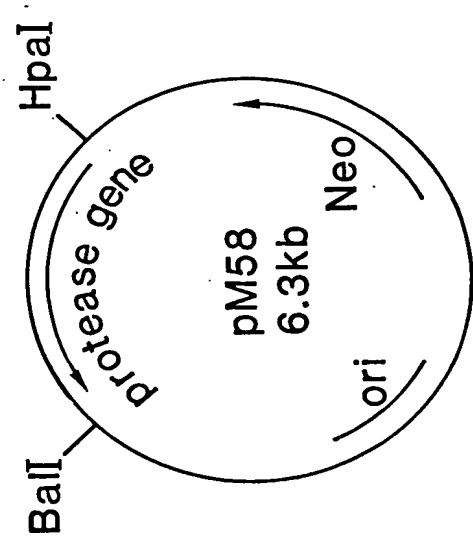
1400 1440
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 plasm.seq. chrom.seq.

1520 1560
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1600 1640
 AGCAGG....CA.100BP....TTCAGTCGTCAAGAGATGCGCAACTACGATAACTCCATTCTTATGGTATGGCTG

1680 1720
 AGAAAATAGCGATTATGAACGTCGTTGGCGGTACATTACCGACGAGCCATATGGGATTGCTGTCCGAAACGGCGACG

EcoRI 1720 HindIII
ACGAATTCACGCGAAAAATCAATGAAATTCTTGCAGACTTAAAGCTT

Figure 6

\times Ball/HpaI

Ligation, Transfection to E. coli JM101.

\times SmaI

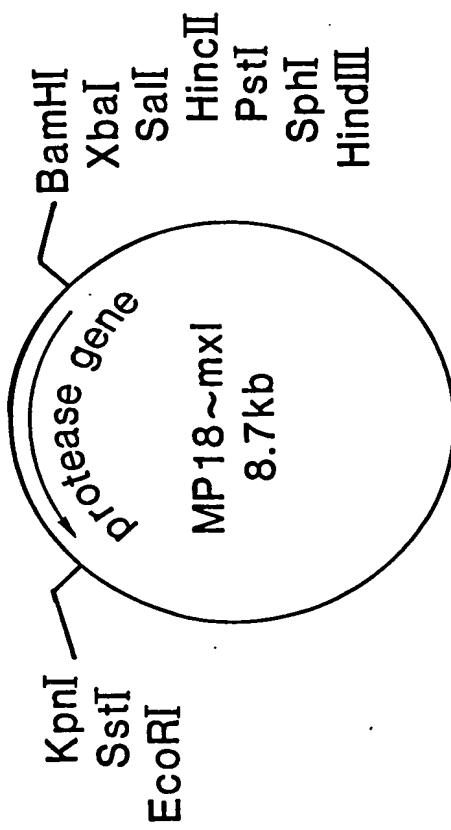


figure 7

Sequence of pBHA-1

11 08 09

AATTCAACCTC GAAAGCAAGC TGATAAACCG ATACAATTAA AGGCTCCTTT TGGAGCCTTT
 30 60

TTTTTGGAG ATTTCACAG TGAAAAAATT ATTATTCGCA ATTCCAAGCT AATTCAACCTC
 90 120

GAAAGCAAGC TGATAAACCG ATACAATTAA AGGCTCCTTT TGGAGCCTTT TTTTTGGAG
 150 180

ATTTCAACG TGAAAAAATT ATTATTCGCA ATTCCAAGCT CTGCCTCGCG CGTTTCGGTG
 210 240

ATGACGGTGA AACCTCTGA CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG
 270 300

CGGATGCAGA TCACGCGCCC TGTAGCGGCG CATTAAGCGC GGCGGGTGTG GTGGTTACGC
 330 360

GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC TCCCTTCGCT TTCTTCCCTT
 390 420

CCTTCTCGC CACGTTCGCC GGCTTCCCC GTCAAGCTCT AAATCGGGGG CTCCCTTTAG
 450 480

GGTTCCGATT TAGTGCTTTA CGGCACCTCG ACCCCAAAAA ACTTGATTAG GGTGATGGTT
 510 540

CACGTAGTGG GCCATCGCCC TGATAGACGG TTTTCGCCCG TTTGACGTTG GAGTCCACGT
 570 600

TCTTTAATAG TGGACTCTTG TTCCAAACTG GAACAACACT CAACCCTATC TCGGTCTATT
 630 660

CTTTTGATT ATAAGGGATT TTGCCGATT CGGCCTATTG GTTAAAAAAAT GAGCTGATT
 690 720

AACAAAATT TAACGCGAAT TTAAACAAAA TATTAACGTT TACAATTGA TCTGCGCTCG
 750 780

GTCGTTCGGC TGCAGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA
 810 840

GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAA GGCCAGGAAC
 870 900

CGTAAAAAGG CCGCGTTGCT GGCCTTTTC CATAGGCTCC GCCCCCTGA CGAGCATCAC
 930 960

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1950 1980
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2010 2040
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2190 2220
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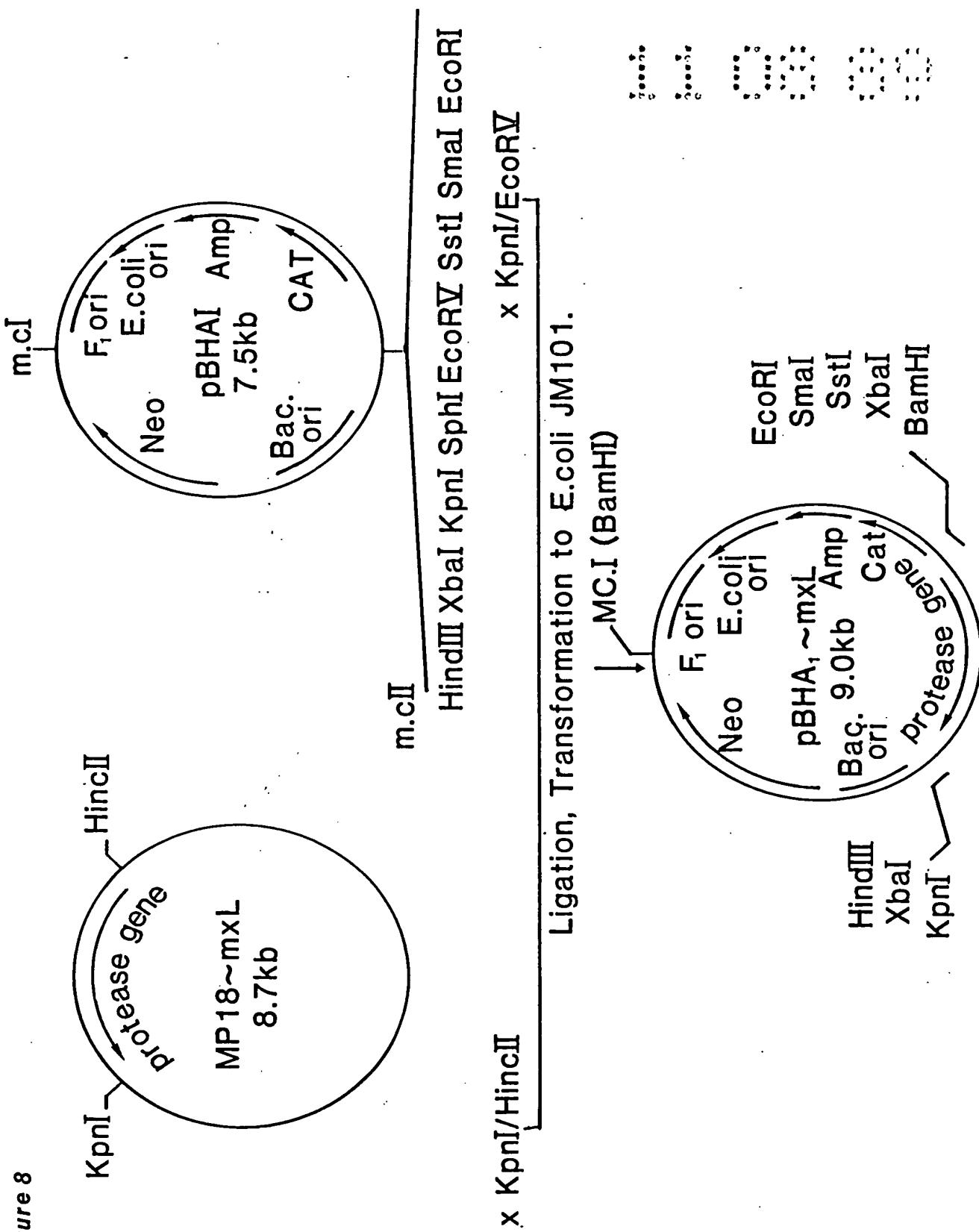
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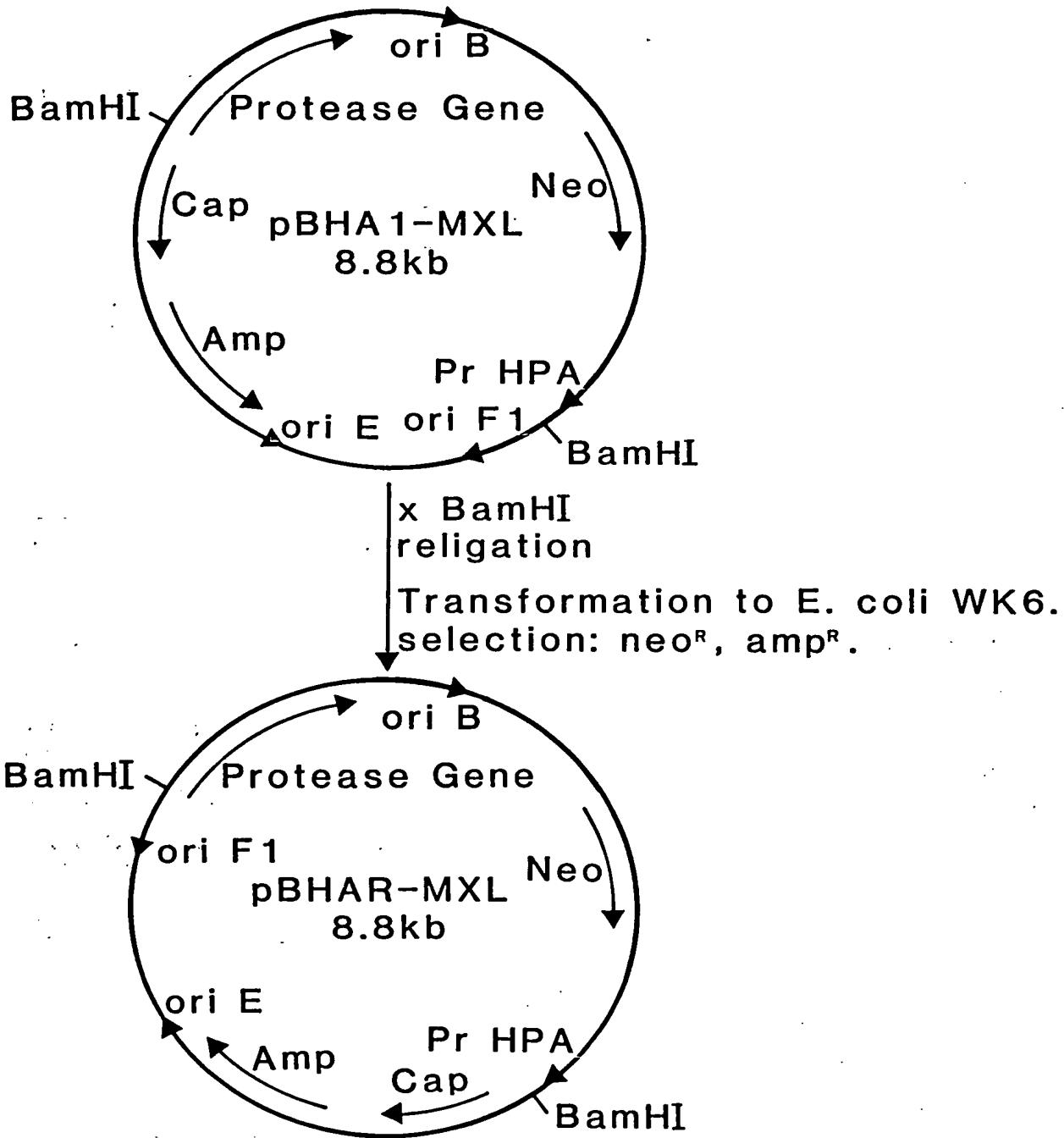
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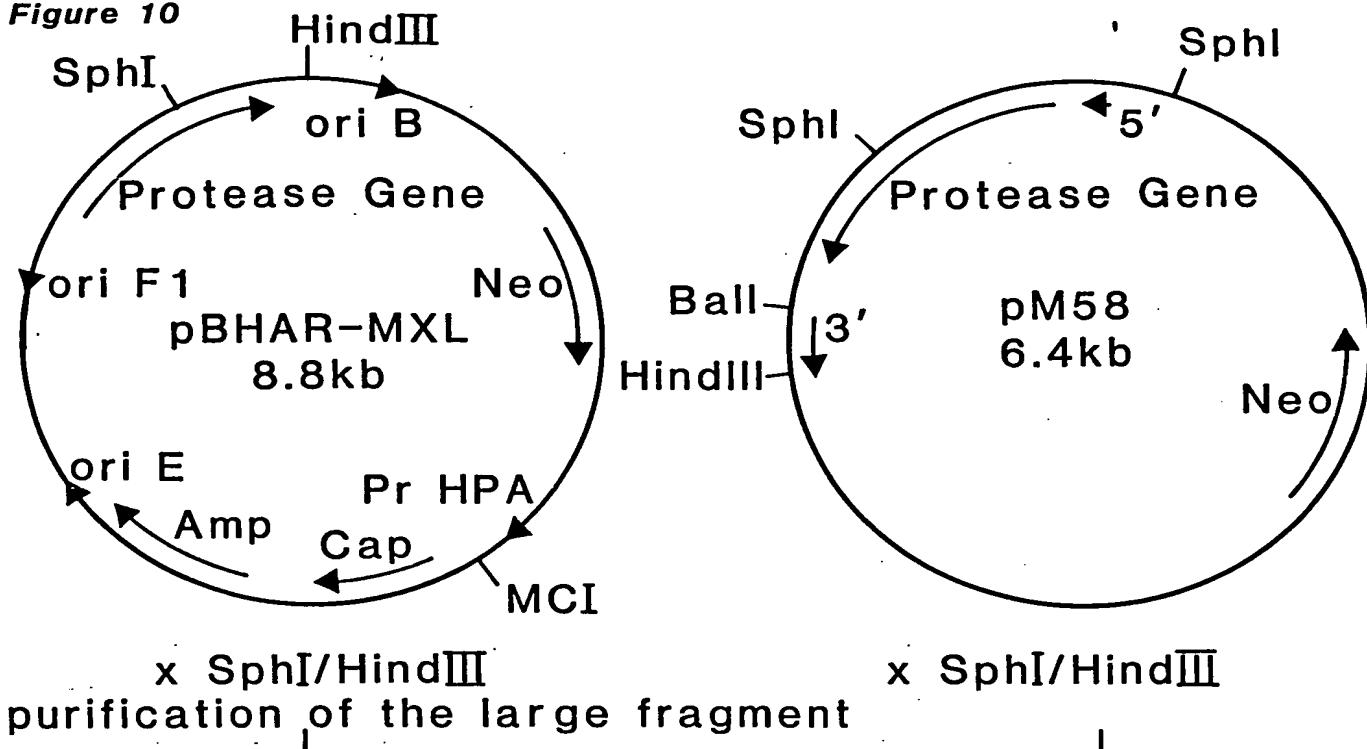
Figure 8

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Figure 9

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Figure 10



Ligation
Transformation
to *B. subtilis* DB104.
selection: prot⁺ neo^r.

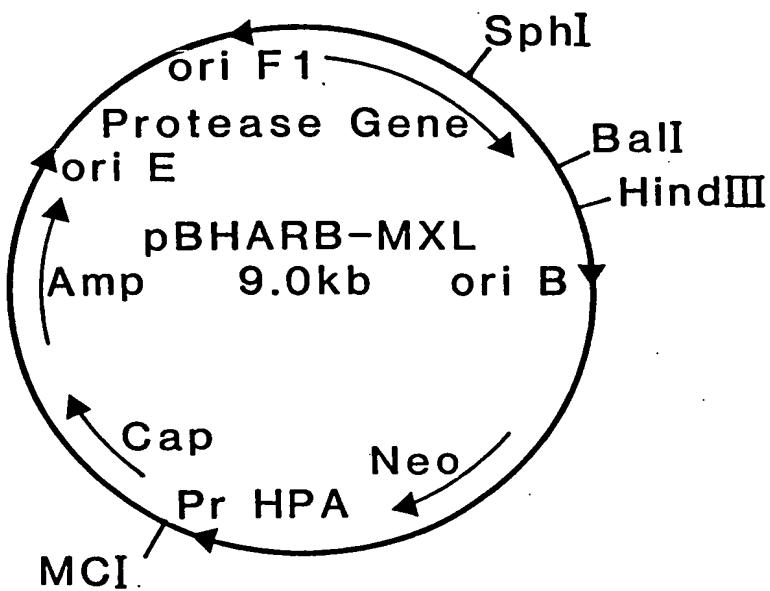


Figure 11

11 00 00

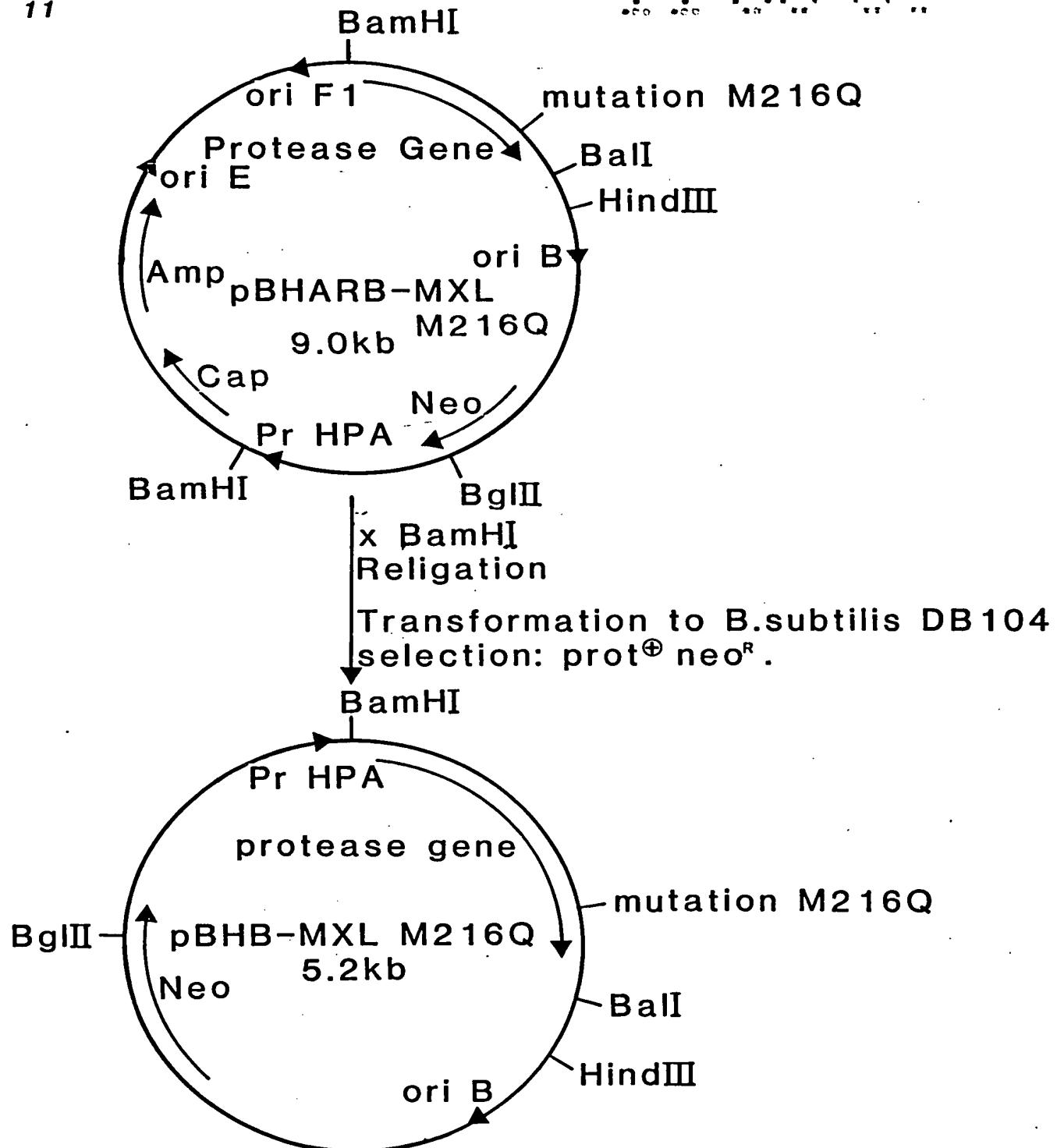
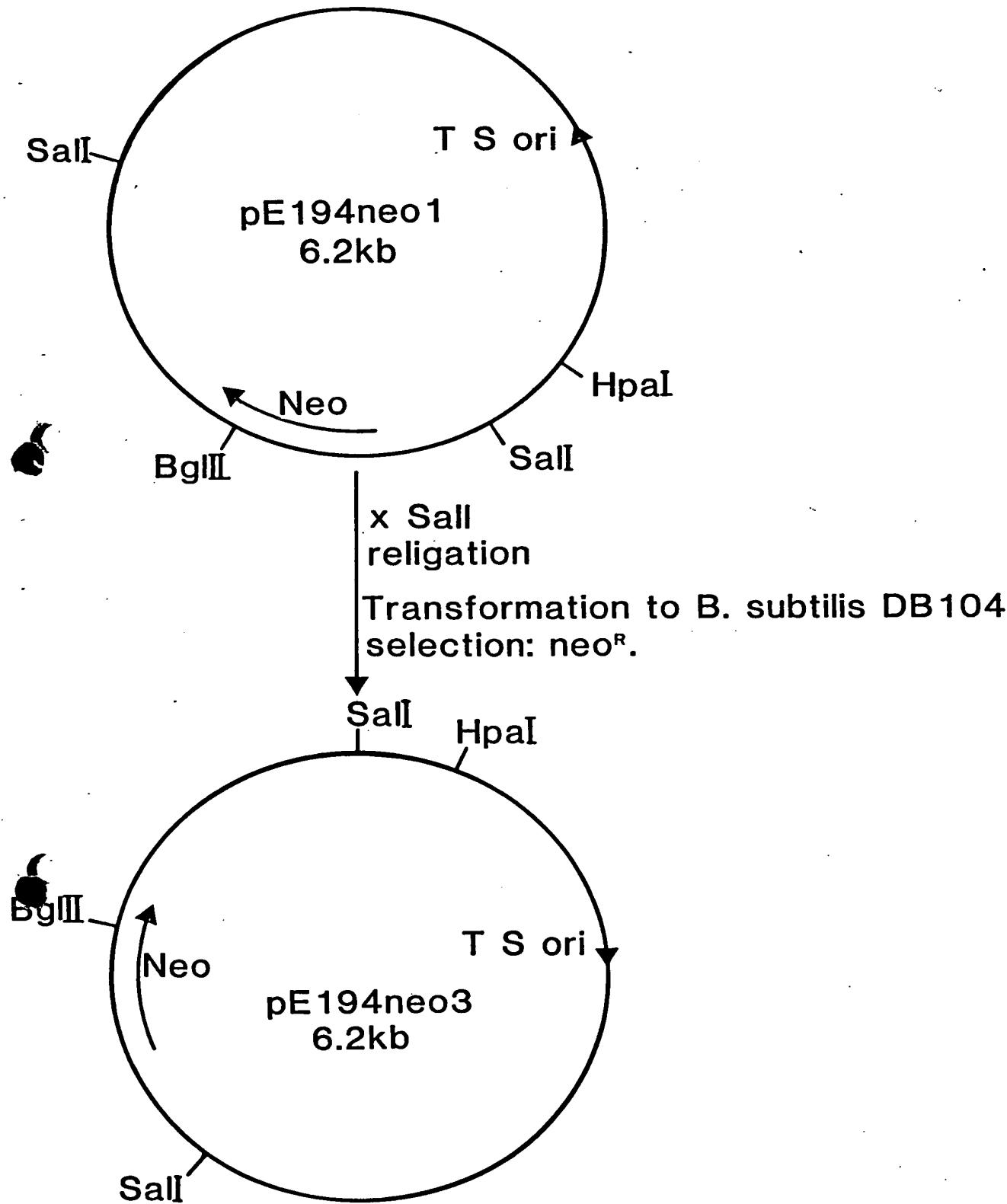


Figure 12

1108 0/0



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Figure 13

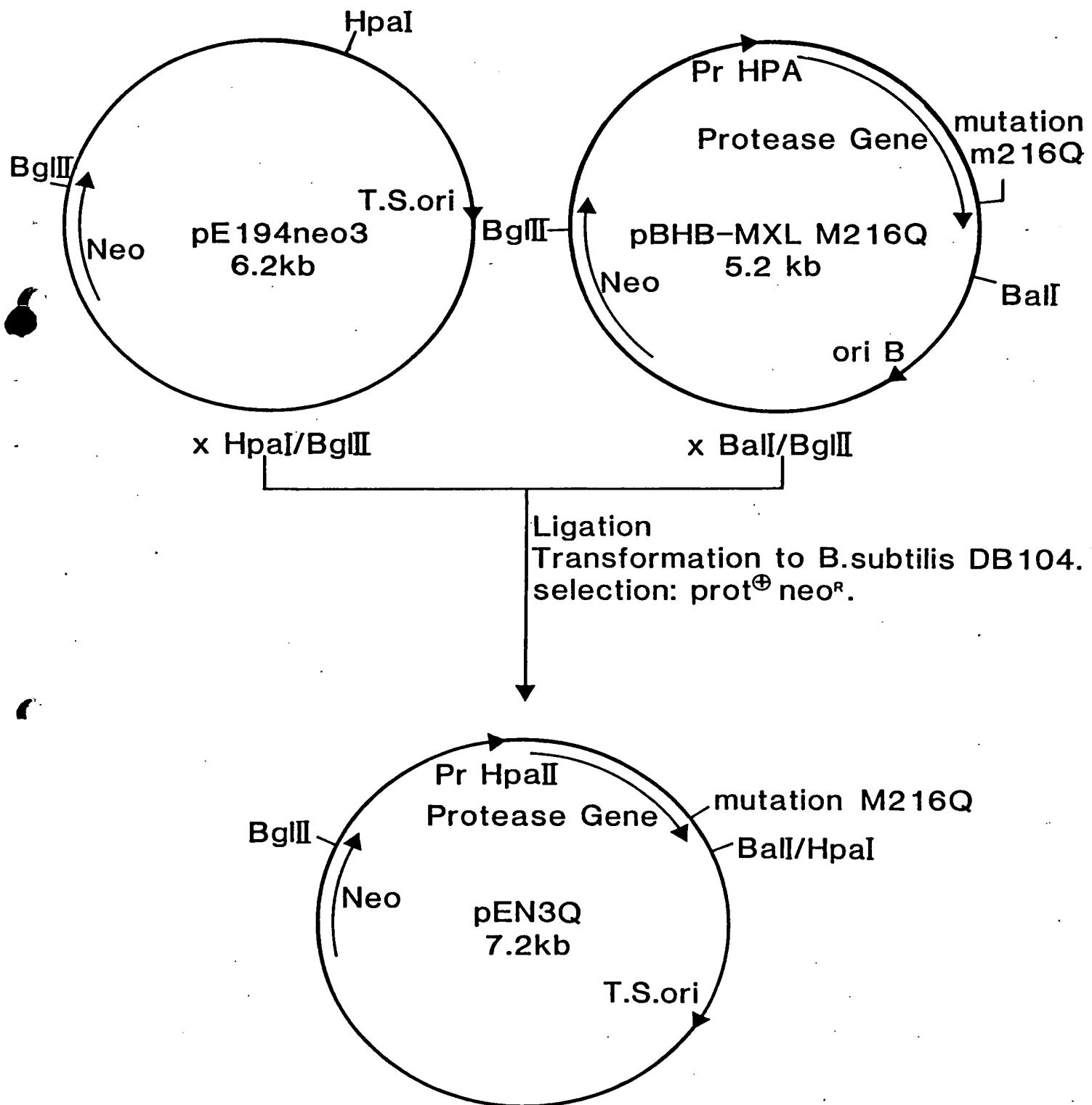


Figure 14

